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Floral Dimorphism and Gynodioecy in *Thymus quinquecostatus* (Lamiaceae)

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The first study of floral dimorphism and the sexual system in natural populations of a Japanese species of *Thymus* is presented. *Thymus quinquecostatus* was previously reported to show a dimorphism in flower size. We investigated floral variation and mode of reproduction to clarify the degree of floral dimorphism and to determine the sexual system. The length of the lower and upper lips, the corolla tube, and the calyx usually showed a monomodal frequency distribution within a population. The length of the outer and inner stamens, however, showed a bimodal distribution. Based on these floral characters the populations were tentatively divided into two discrete morphs: one with long (L) and one with short (S) stamens. The anthers of the L-stamen morph usually produced fertile pollen, while the anthers of the S-stamen morph completely lacked fertile pollen, or poorly developed, abortive pollen grains. Both L- and S-stamen morphs produced fruits and seeds in natural populations. The findings suggest that floral dimorphism in *T. quinquecostatus* may be an example of gynodioecy, as reported in several other species of *Thymus*. Reproductively, pistillate plants show significantly higher fruit and seed production than hermaphrodites, as in some gynodioecious species.

Key words: floral dimorphism, gynodioecy, Lamiaceae, sexual system, *Thymus quinquecostatus*

Gynodioecy, a sexual system in which populations of plants are composed of hermaphroditic and pistillate plants, has been relatively frequently described in various species of the flowering plants (Darwin 1877, Bawa 1980, Richards 1986, 1998, Sugawara & Horii 1995, Williams *et al.* 2000, Hong & Moon 2003). Early estimates reported about 7% of flowering plants to be gynodioecious (Yampolsky & Yampolsky 1922). Gynodioecy was considered to be an unstable, intermediate evolutionary step between hermaphroditism and dioecy (Charlesworth & Charlesworth 1978, Bawa 1980, Bawa & Beach 1981, Richards 1986, 1998). A common feature of gynodioecious species is the occurrence of sexual dimorphism, that is, the hermaphroditic flowers are often larger than the pistillate ones, especially in animal-pollinated, temperate species (Eckhart 1999, Williams *et al.* 2000). Reproductively, relatively higher fruit and seed production in pistillate plants, compared with their hermaphroditic counterparts, has been reported in some gynodioecious species (Uno 1982, Shykoff 1988, Atlan *et al.* 1992, Wolfe & Shmida 1997, Hong & Moon 2003). In other species, however, the pistillate and hermaphroditic plants showed similar seed production, or the pistillate plants had lower seed production (Ashman 1994, Kuhara & Sugawara 2001).

Thymus L. (Lamiaceae), a genus comprising more than 200 species, is distributed from southern Europe to northern Africa and eastern Asia (Mabberley 2008). Members of the genus are small shrubs or perennial herbs. Some are cultivated as garden herbs. The usually insect-pollinated, bilabiate flowers are often differentiated into two sexual morphs (Jalas 1972), with gynodioecy being frequently reported, especially in the European species (Assouad *et al.* 1978, Jalas 1972, Manicacci *et al.* 1998). Moreover, in several gynodioecious species the corolla and calyx show floral dimorphism (Assouad *et al.* 1978, Manicacci *et al.* 1998). In the Japanese *Thymus quinquecostatus* Čelak., two forms have been reported: one with small flowers and the other with large flowers (Kitamura & Murata 1973, Murata 1987, Inoue 1997). The two types of flowers *in T. quinquecostatus* have been suspected as representing sexual dimorphism, as in other gynodioecious species, but floral dimorphism and breeding systems have never been investigated in detail.

In this paper we report on floral dimorphism and breeding systems in three different populations of *Thymus quinquecostatus* in central Honshu, Japan. The reproductive potential and actual fruit and seed production in the different sexual morphs is also reported.

Materials and Methods

Study species and sites

Thymus quinquecostatus Čelak. is a prostrate herbaceous subshrub distributed widely in Japan, Korea, N. China, Manchuria and Sakhalin (Murata & Yamazaki 1993). In Japan, it usually grows in dry stony places on higher mountains from Kyushu to Hokkaido, and occasionally on banks and near the sea in central Honshu and Hokkaido (Kitamura & Murata 1973, Murata 1987). The flowers open from June to August, with many flowers often crowded into a terminal, capitate inflorescence (Fig. 1A, B). A flower comprises a calyx, a sympetalous, 5-lobed, tubular bilabiate corolla with a 2-lobed upper lip and 3-lobed lower lip, four stamens and a pistil (Fig. 1C, D). The stamens are didynamous (Fig. 1E). The superior ovary consistently contains four ovules.

Field investigations were carried out on two populations along the Takasegawa River, Omachi City (Omachi-1 pop., 10m x 10m; Omachi-2 pop., 10m x 5m; both at an altitude of about 700 m), and on one population on Mt. Tateshina, Chino City (Tateshina pop., 10m x 5m; altitude about 1700 m), all in Nagano Prefecture, central Honshu, Japan.

Measurements of floral variation

Floral variation was measured in 64 plants from the Omachi-1 population, 39 plants from the Omachi-2 population, and 25 plants from the Tateshina population. Two inflorescences with fully open flowers were collected from each plant at random and preserved in 70% Ethanol. After fixation, the calyx length (a), lower lip length (b), upper lip length (c), corolla tube length (d), anterior (outer) stamen length (e), and posterior (inner) stamen length (f) of two flowers from each plant were measured, as illustrated in Fig. 1. The mean value of the two flowers was used as representative of each plant.

Pollen stainability and pollen production

Pollen production in each flower and pollen stainability with aniline blue in lactophenol of more than 200 pollen grains per flower was examined for 64 plants from the Omachi-1 population, for 39 plants from the Omachi-2 population, and for 25 plants from the Tateshina population. To evaluate pollen stainability, two flowers per plant were examined and their mean value was used as representative of each plant.

The number of pollen grains per anther was also counted for 60 plants from the Omachi-1 population, for 39 plants from the Omachi-2 population, and for 24 plants from the Tateshina population, and number of pollen grains per flower was calculated.

Fruit and seed set in natural populations

Fruiting under open pollination was examined for all plants within a population, and fruit-set (mature fruit per flower) and seed-set (mature seeds (nutlets) per ovary) were estimated for 30 plants selected randomly from the Omachi-1 population, 13 plants from the Omachi-2 population and 8 plants from the Tateshina population in 2007.

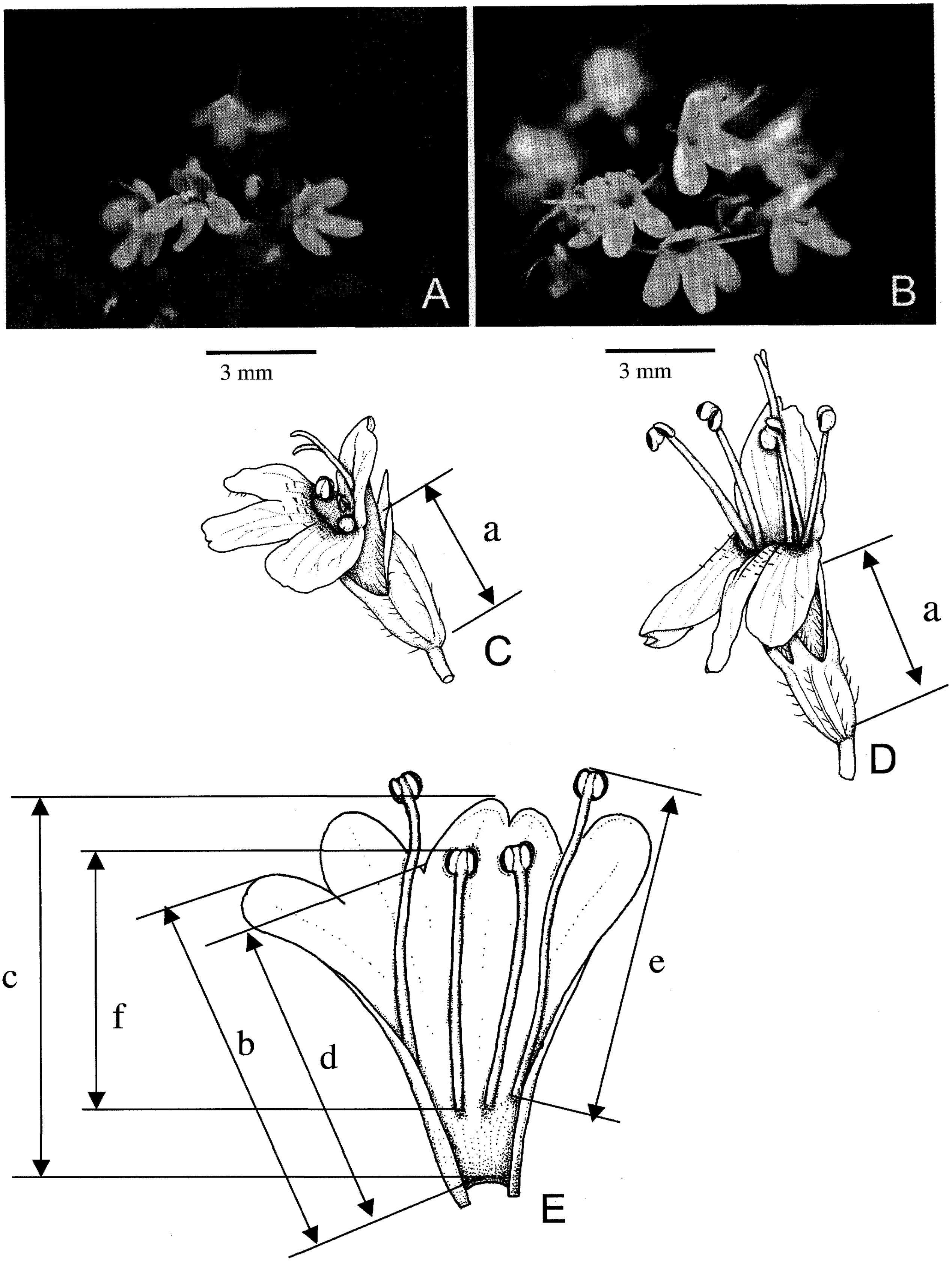

FIG. 1. Flowers of *Thymus quinquecostatus* in the Omachi population, Nagano Prefecture, and their schematic. A & C: Short (S)-stamen morph with style and shorter stamens. B & D: Long (L)-stamen morph with style and longer stamens. E: opened corolla. a, calyx length; b, lower lip length; c, upper lip length; d, corolla tube length; e, outer stamen length; f, inner stamen length.

Artificial pollination experiments

With the plants transplanted from the Omachi-1 population, bagging and artificial self-pollination experiments were carried out to determine the degree of self-compatibility of hermaphroditic flowers in the Biotron NC220 (Nihon Ikakikai Co.). Flower buds selected randomly within each inflorescence were bagged with cellophane. Some of the bagged flowers were left untouched; the others were artificially self-pollinated and rebagged for a month until fruit maturation. Artificial cross-pollination experiments were also carried out between the two different sexual morphs. In the hermaphroditic flowers, emasculated flower buds were bagged and left untouched for 4 days. After elongated papillae appeared on the stigma, indicating maturity, the flowers were artificially cross-pollinated and rebagged for one month until fruit maturation. Details of the number of plants and flowers examined in each pollination experiment are shown in Table 2.

Results

Variation in floral morphology and pollen stainability

Frequency distributions for lower lip length, upper lip length, corolla tube length, outer stamen length, and calyx length obtained from the three populations examined are shown in Fig. 2. Lower lip and corolla tube lengths usually showed a continuous monomodal distribution in each of the populations (Fig. 2O1-A,B; O2-A, B; T-A, B), although a few plants occasionally deviated from the main distribution (Fig. 2O2-A, B). The calyx and upper lip lengths also showed a continuous monomodal distribution in the three populations (Fig. 2O1-D, E; O2-D, E; T-D, E). The outer and inner stamen lengths, however, indicated a bimodal distribution (Fig. 2O1-C, O2-C, T-C). Stamen length therefore divided the plants into two distinct morphs: one with short stamens (tentatively named S-stamen morphs) and the other with long stamens (L-stamen morph).

Plants divided into two morphs based on the stamen length differences showed statistically significant differences in lower lip, upper lip, and corolla tube lengths (Table 1).

Anthers of the S-stamen morphs were presumed to be functionally abortive, so we therefore examined their pollen stainability to compare with L-stamen morphs. Scatter diagrams on the relationship between stamen length and pollen stainability in the three populations are shown in Fig. 3. In the diagrams, each of the two morphs shows obviously different pollen stainabilility, even though there are a few undiscriminated plants in the Omachi-1 and Tateshina populations (Fig. 3A, C; see arrows). The S-stamen morphs usually showed a conspicuously lower value (less than 20 %) in pollen stainability and occasionally completely lacked pollen grains, while the L-stamen morphs showed a higher value (more than 50%) in pollen stainability.

Among the undiscriminated plants in the Omachi-1 and Tateshina populations (Fig. 3A, C), two S-stamen morphs showed a higher value in pollen stainability, and three L-stamen morphs were conspicuously lower in pollen stainability, or had no pollen grains. Those plants appear to be the result of unusual development of the floral organs, and thus we excluded them from subsequent investigations on fruit and seed production.

The number of pollen grains produced by the S-stamen morphs was significantly less than in the L-stamen morphs in the three populations examined (Mann-Whitney's *U*-test, $p < 0.001$ in all populations; Fig. 4).

Fruit and seed set of L- and S-stamen plants in natural populations

All of the L- and S-stamen morphs produced fruit in natural populations. Fruit set percentage of the S-stamen morphs was significantly higher than in the L-stamen morphs in all three populations (Mann-Whitney's *U*-test, $p < 0.01$ in all populations; Fig. 5A). Seed set percentage of the S-stamen morphs was also significantly higher than in the L-stamen morphs (Mann-Whitney's *U*-test, $p < 0.01$; Fig. 5B) in all populations. In the S-stamen morphs, two or three ovules most frequently developed into mature seeds, while in the L-stamen morphs, one ovule most frequently developed into a mature seed (Table 2).

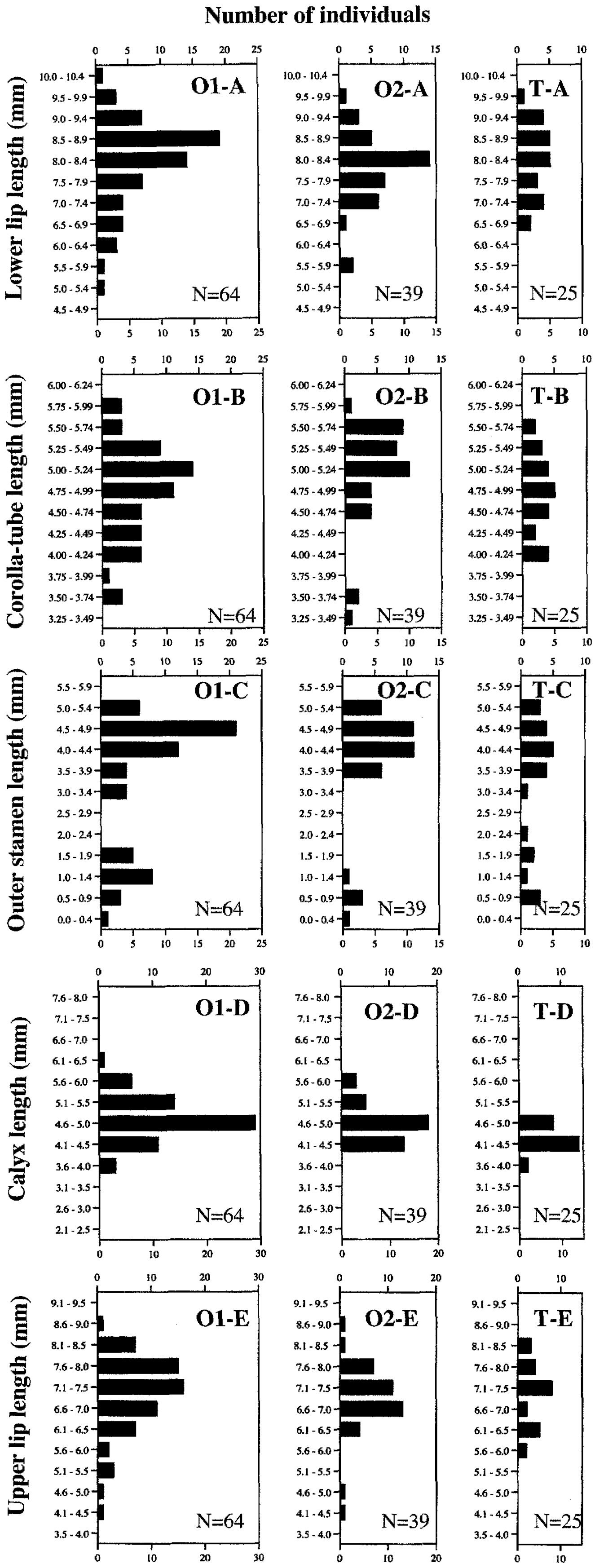

FIG. 2. Frequency distribution of lower lip length (A), corolla tube length (B), outer stamen length (C), calyx length (D) and upper lip length (E) in three populations of *Thymus quinquecostatus*. O1, Omachi-1 population; O2, Omachi-2 population; T, Tateshina population. N, number of individuals examined.

TABLE 1. Measurements of floral characters in long (L) and short (S) stamen morphs in three populations of *Thymus quinquecostatus*.

Population Character	L-stamen morph Mean ± SD	S-stamen morph Mean ± SD	Mann-Whitney *U*-test *P*
Omachi-1 population			
Number of individuals examined	45	15	
Calyx length (mm)	4.87 ± 0.33	4.73 ± 0.37	< 0.05
Lower lip length (mm)	8.61 ± 0.50	7.10 ± 0.52	< 0.001
Upper lip length (mm)	7.51 ± 0.48	6.10 ± 0.51	< 0.001
Corolla-tube length (mm)	5.11 ± 0.43	4.18 ± 0.40	< 0.001
Outer stamen length (mm)	4.39 ± 0.51	1.09 ± 0.26	< 0.001
Inner stamen length (mm)	3.38 ± 0.42	0.69 ± 0.37	< 0.001
Omachi-2 population			
Number of individuals examined	34	5	
Calyx length (mm)	4.81 ± 0.31	4.63 ± 0.29	0.06
Lower lip length (mm)	8.24 ± 0.53	6.39 ± 0.55	< 0.001
Upper lip length (mm)	7.33 ± 0.51	5.60 ± 0.46	< 0.001
Corolla-tube length (mm)	5.11 ± 0.39	3.95 ± 0.51	< 0.001
Outer stamen length (mm)	4.41 ± 0.42	0.79 ± 0.42	< 0.001
Inner stamen length (mm)	3.28 ± 0.37	0.36 ± 0.40	< 0.001
Tateshina population			
Number of individuals examined	16	8	
Calyx length (mm)	4.38 ± 0.09	4.39 ± 0.11	0.07
Lower lip length (mm)	8.47 ± 0.15	7.26 ± 0.26	< 0.05
Upper lip length (mm)	7.31 ± 0.16	6.27 ± 0.26	< 0.05
Corolla-tube length (mm)	4.92 ± 0.10	4.40 ± 0.15	< 0.05
Outer stamen length (mm)	4.27 ± 0.12	1.29 ± 0.21	< 0.001
Inner stamen length (mm)	3.01 ± 0.14	0.73 ± 0.12	< 0.001

Artificial pollination experiments of L-type and S-type flowers

Results of bagging experiments in the L-stamen morph, the artificial self-pollination experiments in L-stamen morph, and the artificial cross-pollination experiments in the L- and S-stamen morphs are shown in Table 3. A significant difference was found among the four treatments in fruit and seed set (Chi-Square test, $\chi^2 = 146.45$, $p < 0.001$ for fruit set; $\chi^2 = 193.74$, $p < 0.001$ for seed set; ref. Preacher 2001). In the bagging experiments with cellophane, the L-stamen morphs produced no fruit, suggesting a dependence on pollinators for fertilization. In the self-pollination experiments, the L-stamen morphs produced fruits and seeds, indicating that the flowers are self-compatible. In the cross-pollination experiments, the S-stamen morphs produce the greatest percentage of fruits and seeds (Table 3).

Discussion

The results indicate that populations of *Thymus quinquecostatus* in Omachi and Tateshina, Nagano Pref., consist of plants of two discrete

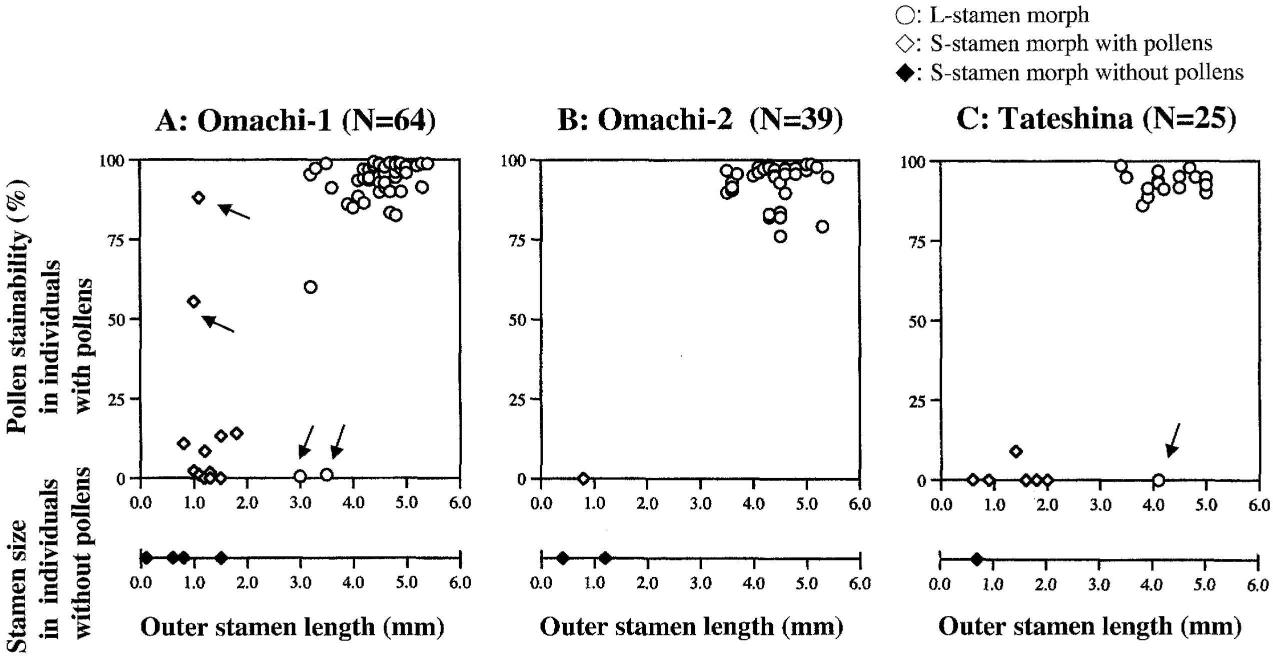

FIG. 3. Relationship between outer stamen length and pollen stainability in individuals with pollen grains, and variation of stamen size in individuals without pollen grains in three populations of *Thymus quinquecostatus*. Arrows indicate undiscriminated plant. N, number of individuals examined.

morphs: short (S) and long (L) stamen morphs. In the S-stamen morphs the filaments of the outer stamen are usually less than 3 mm and the anthers completely lack pollen, or rarely produce fertile pollen. In the L-stamen morphs, the filaments of outer stamens are more than 3 mm long and the anthers usually produce fertile pollen. When we divided the plants into two morphs based on these traits, the differences between the two morphs were obvious in the other floral characters, such as lip length and corolla tube length (see Table 1). The two morphs were highly stable and did not show changes in their floral features for at least two flowering season. In open pollinated natural populations, the two morphs produce fruits and seeds, although fruit and seed production differs significantly between the two morphs. These facts suggest that the S-stamen morphs function only as pistillate plants and the L-stamen morphs as hermaphrodites. Floral dimorphism in *T. quinquecostatus* may therefore represent an example of gynodioecy, and the first report of the phenomenon in a species of *Thymus* in Japan.

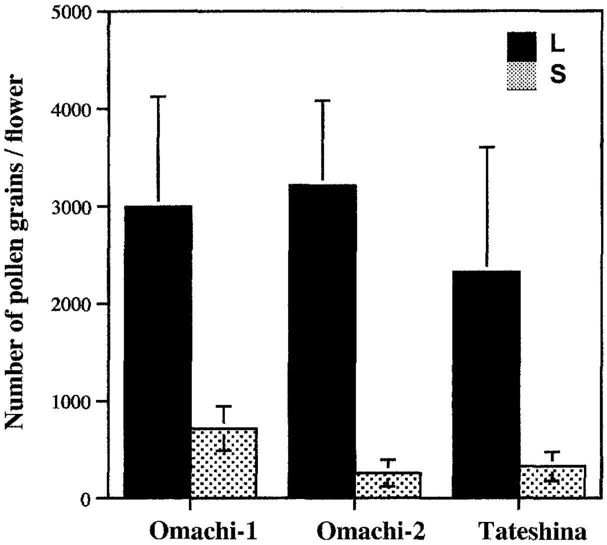

FIG. 4. Number of pollen grains per flower in L- and S-stamen morphs in three populations of *Thymus quinquecostatus*.

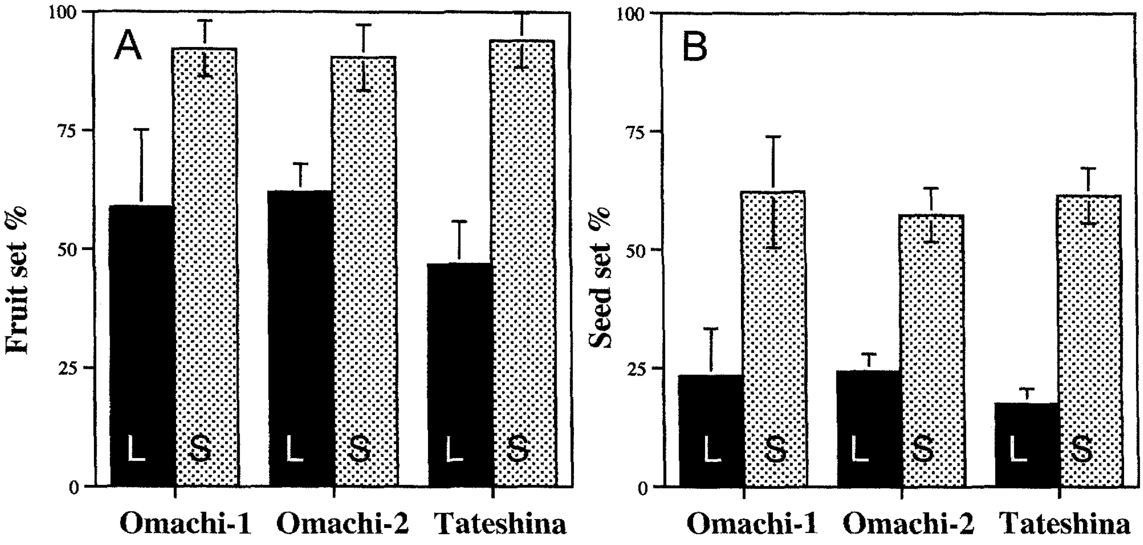

FIG. 5. Fruit (A) and seed (B) production in L- and S-stamen morphs under open pollination in three populations of *Thymus quinquecostatus*.

TABLE 2. Percentage of seed numbers per a fruit examined in long (L) and short (S) stamen morphs in natural populations of *Thymus quinquecostatus*.

Population	Morph	Flower no. (N*)	Percentage of seed numbers within a fruit				
			0	1	2	3	4
Omachi-1	L	900 (N = 18)	41.1	33.7	17.6	6.4	1.2
Omachi-2	L	400 (N = 8)	37.3	39.8	16.3	6.5	0.3
Tateshina	L	250 (N = 5)	53.2	26.8	17.2	2.8	0.0
Omachi-1	S	600 (N = 12)	7.8	12.5	24.0	33.8	21.8
Omachi-2	S	250 (N = 5)	9.6	14.8	30.0	27.6	18.0
Tateshina	S	150 (N = 3)	6.0	12.7	28.0	36.0	17.3

*Number of individuals examined.

TABLE 3. Pollination experiments on long (L) and short (S) stamen morphs in the Omach-1 population of *Thymus quinquecostatus*.

Treatment	N1*	N2**	N3***	Fruit set (%)	Seed set (%)
Bagged L	3	28	0	0.0	0.0
Artificial self-pollination L	6	190	66	34.7	28.4
Artificial cross-pollination L	7	268	126	47.0	34.7
Artificial cross-pollination S	3	130	122	93.8	62.1

*N1: number of individuals examined. **N2: Number of flowers examined in total. ***N3: number of flowers fruited.

It is usual in some gynodioecious species that pistillate plants produce more seeds in comparison with hermaphroditic plants, either from avoidance of selfing in the pistillate plants or the reallocation of resources into pistillate function (Uno 1982, Richards 1986, 1997, Charlesworth & Charlesworth 1978, 1987, Kikuzawa 1989, Sakai *et al.* 1989, 1997, Delph 1990, Maki 1993). In natural populations of *Thymus quinquecostatus* observed here, the pistillate S-stamen morphs produced fruits and seeds in greater amounts than did the hermaphroditic L-stamen morphs, as in several other gynodioecious species of *Thymus* (Assouad *et al.* 1978, Atlan *et al.* 1992, Manicacci *et al.* 1998). Although we did not examine the relative importance of inbreeding depression and resource allocation in seed production, there is some evidence that less outcrossing in hermaphrodites than in pistillate plants may be responsible for the differences in seed production. Firstly, hermaphrodites simultaneously open many flowers on the same plant, which thereby may result in a higher incidence of self-pollination in nature. Secondly, artificial self-pollination of hermaphroditic flowers results in a significantly lower seed production than does cross-pollination. These facts suggest that lower seed production in hermaphrodites may be at least partly due to the inbreeding effect on seed production.

Although we did not examine the quality of germination, seedling survival, and growth of juveniles in the offspring, sexual differences in those characters have been demonstrated to play a role in the maintenance of the gynodioecious breeding system in other self-compatible species (Assouad *et al.* 1978, Sakai *et al.* 1989, 1997, Wolfe & Shmida 1997). Further investigation on the above characters throughout a lifetime is necessary to obtain reliable estimates of the inbreeding effects in the Japanese species of *Thymus*.

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